MAJOR ARTICLE

Bacteriophages Can Treat and Prevent *Pseudomonas aeruginosa* Lung Infections

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Antibiotic-resistant bacteria threaten life worldwide. Although new antibiotics are scarce, the use of bacteriophages, viruses that infect bacteria, is rarely proposed as a means of offsetting this shortage. Doubt also remains widespread about the efficacy of phage therapy despite recent encouraging results. Using a bioluminescent *Pseudomonas aeruginosa* strain, we monitored and quantified the efficacy of a bacteriophage treatment in mice during acute lung infection. Bacteriophage treatment not only was effective in saving animals from lethal infection, but also was able to prevent lung infection when given 24 h before bacterial infection, thereby extending the potential use of bacteriophages as therapeutic agents to combat bacterial lung infection.

Pulmonary infections are one of the major causes of mortality worldwide. Each year it is estimated that ~2 million children <5 years old die of acute respiratory infections [1]. Furthermore, the number of bacterial infections is probably increasing because of resistance to antibiotics. Opportunistic pathogens are becoming increasingly resistant to multiple antibiotics, which urges us to seek other therapeutic approaches, since new antibacterial compounds are scarce [2].

Phage therapy is one of several potential therapeutic approaches and has been considered since the late 1980s [3–6]. Bacteriophages are viruses that target and infect only bacteria. Since the middle of the 20th century, studies of bacteriophages have helped to elucidate fundamental cellular processes, and bacteriophages are now among the best known biological entities at the molecular level. Two important factors support the consideration of phage therapy: (1) it has been used for decades in eastern Europe and is still used today to treat infections in humans [7, 8]; and (2) bacteriophage biology is much better understood today than it was in the mid-20th century, when it was overlooked in favor of antibiotics [9–11]. Three main characteristics distinguish bacteriophage therapy from antibiotic therapy: (1) bacteriophages multiply at the infection site; (2) they target only specific bacteria, with no effect on commensal flora; and (3) they can adapt to resistant bacteria.

The bacterium *Pseudomonas aeruginosa* causes acute pneumonia with a high mortality rate in immunocompromised patients; in patients with cystic fibrosis (CF), it triggers chronic inflammation that leads to destruction of the lungs [12]. Interestingly, a large number of *P. aeruginosa* bacteriophages have been observed, and 38 complete genomes are now available in the National Center for Biotechnology Information databases [13]. However, few papers have described the use of *Pseudomonas* bacteriophages in therapy experiments, and to our knowledge there have been no tests on a lung infection model [14–16]. In this work, we aimed to de-

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termine whether a natural bacteriophage isolated from the environment could be suitable for therapeutic use in an animal lung infection model. We used a bioluminescent *P. aeruginosa* strain to record a real-time view of the lung infection, thus allowing us to monitor the spatial and temporal development of infections in small live animals [17]. This made it possible to quantify the efficacy of bacteriophage treatment in live animals, which unambiguously demonstrates its potential to treat bacterial lung infections.

MATERIALS AND METHODS

P. aeruginosa *strains.* The bioluminescent PAK strain (PAK lumi) used in this study has been described elsewhere [18] and was kindly provided by R. Ramphal (Gainesville, FL). We obtained 10 primary colonization strains and 10 chronic colonization strains of *P. aeruginosa* from the French cystic fibrosis strain collection center (P. Plésiat, Grenoble, France).

Bacteriophage isolation, preparation, and characterization. The PAK-P1 bacteriophage was isolated from sewage water as described in the Appendix, which appears only in the online version of the *Journal*. Large-scale preparation of bacteriophages was performed from 1 L of liquid culture as described by Boulanger [19]. For the animal experiments, bacteriophages prepared by cesium chloride ultracentrifugation were diluted in phosphate-buffered saline (PBS).

Plaque assays were performed to determine plating efficacy on clinical strains of *P. aeruginosa* according to standard protocols.

Electron microscopic analysis was performed on cesium chloride bacteriophage preparations [20], and observations were made after uranyl acetate staining with a JEOL 1200 EXII electron microscope.

Genome sequencing (20× coverage) was performed by Eurofins using 454 technology on DNA obtained by means of standard procedures. The complete genome sequence of the PAK-P1 bacteriophage is accessible in GenBank (accession no. GQ422154).

The microsequence of the major capsid protein was determined by the Institut Pasteur microsequencing facility after the separation of whole bacteriophage proteins on a sodium dodecyl sulfate gel followed by in-gel trypsin digestion. Peptides were identified only after adding the genomic sequence of the PAK-P1 bacteriophage to the peptide identification database. The 38 fully sequenced *P. aeruginosa* bacteriophage genomes are available on the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=10239&type=6&name=Phages).

Ethics statement. Mice (8-week-old Balb/c males) were supplied by the Centre d'élevage R. Janvier and housed in an animal facility in accordance with Institut Pasteur guidelines and in agreement with European recommendations. Food and drink were provided ad libitum.

Animal infections. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine-xylazine before being infected. The infectious dose was 1×10^7 luminescent bacteria resuspended in 50 μL of PBS. In curative experiments, 2 h after bacterial instillation the bioluminescence was recorded and 30 μL of bacteriophages were applied intranasally while the mice were still asleep (isofluorane inhalation). In preventive experiments, 24 h before infection the animals received intranasally 30 μL of bacteriophages or PBS while under a light anesthesia by means of isofluorane inhalation.

Luminescence measurements. Photon emission of the luminescent bacteria in the lungs of infected mice was quantified using an IVIS 100 imaging system (Xenogen Biosciences). After infection, mice were anesthetized by means of isofluorane inhalation, and the luminescence of the bacteria was recorded by means of a charge-coupled device camera coupled to the LivingImage software package (version 3.1; Xenogen). A digital false-color photon emission image was generated, and photons were counted within a constant-defined area corresponding to the surface of the chest and encompassing the whole lung region. All data were normalized by subtracting the average background level obtained from noninfected animals. Photon emission was expressed as photons/s/cm^2/steradian. Images shown for each experiment were directly taken from the LivingImage software, in which the color scale was identical for each individual image.

Bronchoalveolar lavage and inflammation analyses. Bronchoalveolar lavages (BALs; 4 lavages of 0.5 mL each) were performed at the indicated time points after infection following euthanasia (intraperitoneal administration of pentobarbital). One part of the BAL fluids was centrifuged at 1400 rpm for 10 min, and then murine cytokine concentrations were determined using DuoSet enzyme-linked immunosorbent assay kits (R&D Systems). Another part of the BAL fluids was centrifuged at 6000 rpm for 10 min, and supernatants were diluted and spotted on plates overlaid with the PAK strain to determine the amount of free bacteriophages, whereas pellets were resuspended in PBS and serial dilutions were plated on Luria-Bertani agar plates to determine viable bacterial counts.

Statistical analysis. *P* values were calculated with the unpaired *t* test, using XLStat software (version 2008.7; Addinsoft). Data are given as mean ± standard error of the mean.

RESULTS

Dose- and time-dependent effect of bacteriophages on infected mice. We isolated from sewage water a bacteriophage specific to the PAK strain of *P. aeruginosa*, which we named PAK-P1 (see below for characterization). To ascertain the effect of the

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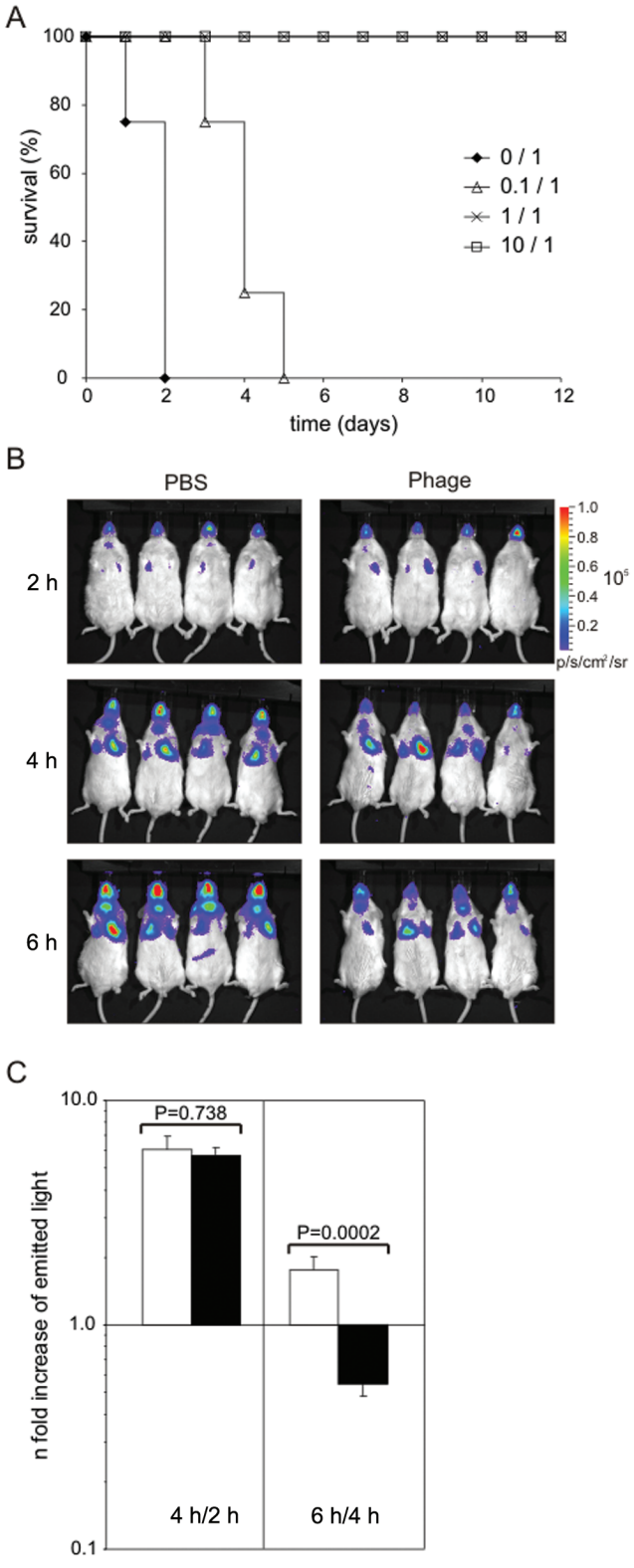

PAK-P1 bacteriophage on infection in a live animal lung infection model, mice were infected with the bioluminescent PAK strain and then treated with bacteriophages. Both bacteria and bacteriophages were administered by means of intranasal instillation. After a preliminary experiment that showed that the PAK-P1 bacteriophage was active in vivo by delaying the death of highly infected animals (data not shown), an experiment was designed to determine the amount of this bacteriophage required to fully cure infected mice (Figure 1*A*). Although non–phage-treated mice died within 48 h after inoculation with PAK (most of them were still alive after 24 h), mice treated with bacteriophages in a phage-to-bacterium ratio of 1:10 died within 5 days after inoculation with PAK. Mice treated with higher bacteriophage-to-bacterium ratios (1:1 and 10:1) survived until the end of the experiment (12 d). In 2 independent experiments, 100% of the animals treated with the 10:1 dose survived, whereas 100% and 80% of the animals treated with the 1:1 dose survived, which led us to choose the bacteriophage-to-bacterium ratio of 10:1 as the standard dose for future experiments.

An active bacteriophage was required to cure the animals. Mice treated with a solution of heat-killed PAK-P1 bacteriophage 2 h after infection died at the same rate as did untreated animals (data not shown). Moreover, to determine whether PAK-P1 bacteriophage treatment was harmless to animals, an intranasal dose that was 10 times higher than that defined above as the standard dose was administered to a group of mice ($n = 8$), and their behavior was monitored for 10 days. These mice did not show erratic behavior, their fur remained regular, and they gained weight, which suggests that the bacteriophage solution had no adverse affect on them.

The rate at which the PAK-P1 bacteriophage was able to eliminate bacteria in vivo was estimated by quantifying the light

Figure 1. Effect of bacteriophage treatment on deadly infection in mice. *A*, Survival curves of infected animals treated with phosphate-buffered saline (PBS) or bacteriophages at indicated bacteriophage-to-bacterium ratios. The amount of bacteria required to induce a deadly lung infection in Balb/c mice by way of intranasal instillation (modified from Balloy et al [35]) was set to 1×10^7 bacteria, because we found that 100% of mice survived challenge by 5×10^6 bacteria for up to 4 days and that a dose of 1.5×10^7 bacteria was 100% lethal within 24 h. *B*, Example of time-course images of mice infected with bioluminescent *Pseudomonas aeruginosa* and treated with PBS (*left*) or treated with the PAK-P1 bacteriophage at a bacteriophage-to-bacterium ratio of 10:1 (*right*). *C*, Quantification of emitted light given as a ratio between 2 time points (*left*, 4 h/2 h; *right*, 6 h/4 h). The amount of light emitted from the chest area of infected mice ($n = 12$ from 2 independent experiments) treated with PBS (*white bars*) or treated with the PAK-P1 bacteriophage at a phage-to-bacterium ratio of 10:1 (*black bars*) was quantified. Bars show the mean, and error bars show the standard error. p/s/cm^2/sr, photons/s/cm^2/steradian.

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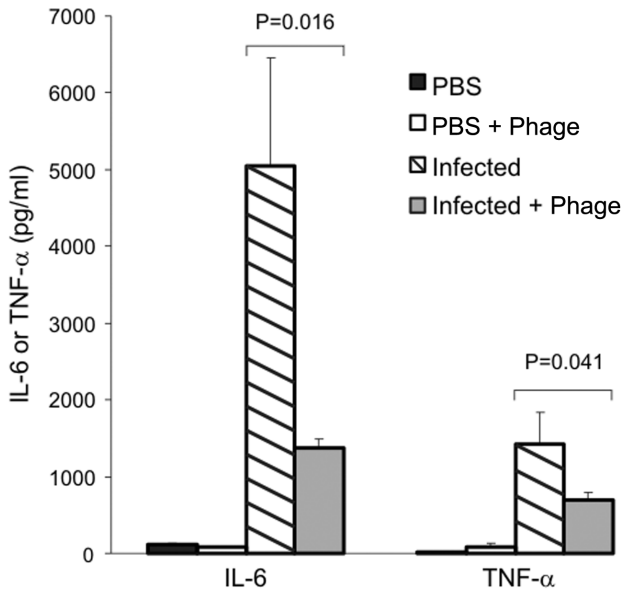

Figure 2. Reduction of inflammation by bacteriophage treatment. Cytokine levels were measured in bronchoalveolar lavages of mice ($n = 4$) 24 h after instillation of phosphate-buffered saline (PBS) (*black bars*), PBS and PAK-P1 bacteriophage (*white bars*), bacteria with PBS 2 h later (*hatched bars*), or bacteria with PAK-P1 bacteriophage 2 h later (*gray bars*). Bars show the mean, and error bars show the standard error. IL-6, interleukin 6; TNF-α, tumor necrosis factor α.

emitted by the bioluminescent bacteria in live animals during the first hours of infection (Figure 1*B*). Animals were first inoculated with the PAK strain and then with the PAK-P1 bacteriophage 2 h later. Between 2 and 4 h after bacterial infection, the amount of light emitted from the phage-treated mice and the amount of light emitted from the non–phage-treated mice showed no statistically significant difference, demonstrating that the initial evolution of the infection was similar in both groups during the first 4 h (Figure 1*C*). However, at the 6 h time point (ie, 4 h after the bacteriophage was administered), the amount of light emitted from phage-treated mice was statistically significantly reduced compared with the amount of light emitted from the non–phage-treated mice, suggesting rapid killing of bacteria by bacteriophages. At 24 h after the start of infection, phage-treated mice showed no or only weak spots of light, whereas non–phage-treated mice were dead or highly luminescent (data not shown). This suggests that the amount of bacteria is strongly reduced in phage-treated animals, and thus they can survive lethal bacterial challenge.

We measured the bacterial load and bacteriophage amounts in BALs after 24 h of infection. As expected, no bacteriophage was detected in BALs of non–phage-treated mice, and the average amount of bacteria was 1.6×10^8 bacteria/mL ($n = 2$). In contrast, only 1.5×10^2 bacteria/mL, together with 2×10^7 bacteriophages/mL, was recovered from BALs of phage-treated mice ($n = 4$). In comparison, PAK-P1 bacteriophage was detected at a concentration of 3.1×10^6 bacteriophages/mL in BALs of uninfected phage-treated mice ($n = 4$), which confirmed that bacteriophages multiplied inside the lungs of both infected and uninfected bacteriophage-treated animals.

In the above experiments, bacteriophage solutions were instilled 2 h after inoculation with the bacteria, but this time point might not correspond to an infection status. To gain more insight about this infection status, we measured the level of lactate dehydrogenase (an enzyme released when the cell in-

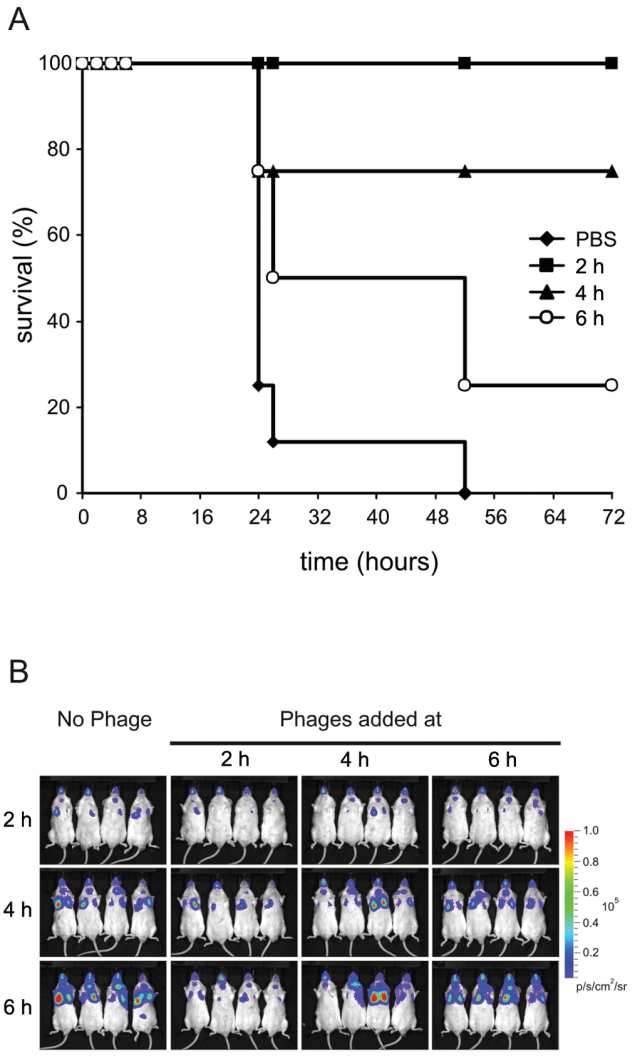

Figure 3. Time-course images of bacteriophage treatment. *A*, Survival curves of infected mice treated with phosphate-buffered saline (PBS) (*diamonds*) or with the PAK-P1 bacteriophage at a phage-to-bacterium ratio of 10:1 at 2 h (*squares*), 4 h (*triangles*), or 6 h (*circles*) after the infection was initiated. *B*, Images corresponding to the early time points of the experiment presented in panel *A*. p/s/cm^2/sr, photons/s/cm^2/steradian.

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Table 1. Efficacy of Plating of the PAK-P1 Bacteriophage on *Pseudomonas aeruginosa* Strains from Patients with Cystic Fibrosis

	Efficacy of plating relative to bioluminescent PAK strain, %									
	Clinical strain									
Source of clinical strain	1	2	3	4	5	6	7	8	9	10
Patients with primary infection	0	0	0	100	100	100	10	100	100	0
Patients with chronic infection	0	0.1	0	0	0.001	0	0	0.0001	0	0

tegrity is damaged) present in BALs 6 h after the infection was initiated. Compared to controls (PBS-treated mice and mice treated with bacteriophage only), levels of lactate dehydrogenase in both non–phage-treated mice and phage-treated mice were 3.7-fold and 3.1-fold higher, respectively, showing clearly that at this time point the lung damage was equivalent in these 2 groups of infected animals (data not shown).

Reduction of inflammatory response after bacteriophage treatment. We hypothesized that if bacteriophages are able to kill bacteria in vivo, leading to a reduction in the amount of these bacteria in the lungs, then the inflammatory response (the first line of host defence against invading pathogens) should be lower. At 24 h after infection the levels of 2 inflammatory markers, tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6; known to be induced by a bacterial challenge) were evaluated in BALs from non–phage-treated mice and phage-treated mice (Figure 2) [21–23]. Both IL-6 and TNF-α increased in PAK-infected animals (both those treated with bacteriophage and those not treated with bacteriophage) compared with noninfected controls, confirming that the infection had started in both groups. However, these levels were statistically significantly reduced in the bacteriophage-treated group in comparison with the untreated group, confirming that reduction of the number of bacteria by bacteriophage treatment attenuated the host inflammatory response. At 48 h after infection, IL-6 and TNF-α levels returned to the baseline values in the phage-treated group (data not shown). It should also be noted that the levels of IL-6 and TNF-α were as low in the noninfected animals treated with the bacteriophage solution as the levels in the animals that received PBS solution, which shows that bacteriophages alone did not seem to stimulate an inflammatory response (Figure 2).

Timing of the bacteriophage treatment. We next determined the maximum possible delay of bacteriophage treatment to maintain an animal survival rate of 100% by administering treatment at 2, 4, or 6 h after infection. Although 100% of mice survived in the group treated with phages 2 h after infection, at 24 h only 75% of mice were still alive in the groups treated 4 or 6 h after infection. At 72 h, survival was close for the 2 h group (100% of mice) and 4 h group (75% of mice) but had dropped to 25% for the 6 h group (Figure 3*A*). These results could be anticipated on examination of the bioluminescence images at early time points (Figure 3*B*). These experiments showed that bacteriophage treatment had to be given 2 h after infection to reach 100% survival in infected animals.

Bacteriophage efficacies on clinical strains. To estimate the host range of the PAK-P1 bacteriophage against clinical strains, we determined its efficacy against a panel of 20 *P. aeruginosa* strains isolated from patients with cystic fibrosis. We tested 10 strains from patients with primary colonization and 10 strains from patients with chronic colonization. The PAK-P1 bacteriophage was able to effectively lyse 50% of the primary colonization strains, but it only moderately lysed 10% of the chronic ones (Table 1).

No development of infection in bacteriophage-treated mice. Because bacteriophages administered in uninfected animals persisted in reasonable amounts inside the lungs for at least 24 h, we tested whether bacteriophage pretreatment would prevent subsequent infection. Two groups of mice, 1 group treated intranasally with 1×10^8 PAK-P1 bacteriophages and 1 group treated intranasally with buffer, were infected 24 h later with 1×10^7 bacteria. At 2 h after bacterial inoculation, the amount

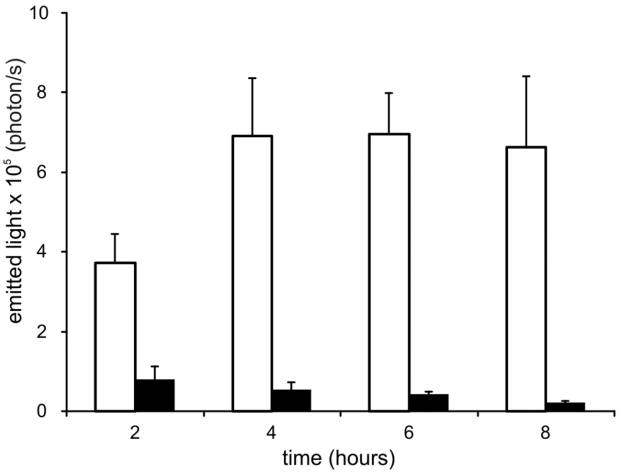

Figure 4. Efficacy of bacteriophage pretreatment 24 h before infection. Shown is the time course of light emitted (in photons/s) from the chest area of mice pretreated with phosphate-buffered saline (PBS) (*white bars*) or with PAK-P1 bacteriophage (*black bars*) 24 h before infection with *Pseudomonas aeruginosa* ($n = 4$ for each group). Bars show the mean, and error bars show the standard error.

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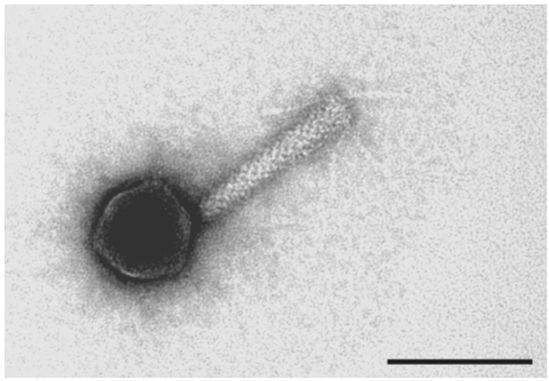
A



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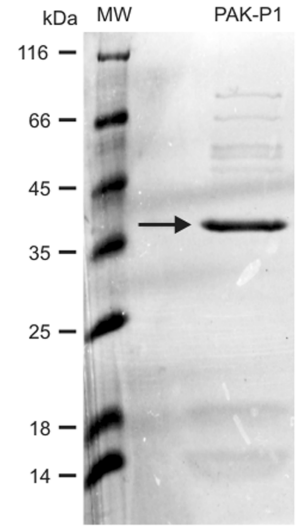

C

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>PAK-P1:Major capsid protein
MANTRSYLNDGQFYIADQTENLLIIPNTWTLVENMGVFTSEGVTQNTVQF
EEIETRYGLVKDAIRGTRHQVASDQRRQLRAFAIPHFNQDDYITPEDIQG
KRAFGADREETLNEVRARKLETIRRNWANTAEVASVSAIVTGKSYAPAGT
IEYDWYDLMGKTRKVVGFDLTNPTADVMGKTEEIFVHMQDNSQDGLIRGD
FVALCSPEFFTALINHPSIKEFYKAYQASPQYWRERLTARGLDLRFREFY
FGNIHFIEYRGVDPYGNRLIPAGDAYFIPTDSGDLFARYFGPGSTFDDLG
TLGKELYATERMAEDRRSILIETESNFIHVLRRPQMIVRGTVNA
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D

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 GENE ID: 2744044 Felix01p112 | major capsid protein
[Enterobacteria phage Felix 01]

 Score =  135 bits (340),  Expect = 6e-30, Method: Compositional matrix adjust.
 Identities = 102/354 (28%), Positives = 170/354 (48%), Gaps = 30/354 (8%)

Query  12   QFYIADQTENLLIIPNTWTLVENMGVFTSEGVTQNTVQFEEIETRYGLVKDAIRGTRH-Q  70
            +F++AD T  +  IPNT+  + N+G+F S  +TQ T   +  +    L+    R +R  +
Sbjct  9    RFFLADLTGEVQSIPNTYGYISNLGLFRSAPITQTTFLMDLTDWDVSLLDAVDRDSRKAE  68

Query  71   VASDQRRQLRAFAIPHFNQDDYITPEDIQGKRAFGADREETLNEV-RARKLETIRRNWAN  129
             ++ +R +  +F + +F + + ITP++IQG R  G   E T   V RA+KL  IR  +
Sbjct  69   TSAPERVRQISFPMMYFKEVESITPDEIQGVRQPGTANELTTEAVVRAKKLMKIRTKFDI  128

Query  130  TAEVASVSAIVTGKSYAPAGTIEYDWYDLMGKTRKVVGFDLTNPTADVMGKTEEIFVHMQ  189
            T E   + A+  GK     GT+  D Y      +K + FDL NP AD+    EE+ +HM+
Sbjct  129  TREFLFMQAL-KGKVVDARGTLYADLYKQFDVEKKTIYFDLDNPNADIDASIEELRMHME  187

Query  190  DNSQDG-LIRGDFVALCSPE-FFTALINHPSIKEFYKAYQASPQYWRERL------TARG  241
            D ++ G +I G+ + +     FF+ L  HP I++ Y A Q +P  W++          A G
Sbjct  188  DEAKTGTVINGEEIHVVVDRVFFSKLTKHPKIRDAYLA-QQTPLAWQQITGSLRTGGADG  246

Query  242  LDLRFREFYFGNIHFIEYRG--------------VDPYGNRLIPAGDAY---FIPTDSGD  284
            +      FY+G + F++Y G              +D   +  +  G A+    +  ++ +
Sbjct  247  VQAHMNTFYYGGVKFVQYNGKFKDKRGKVHTLVSIDSVADT-VGVGHAFPNVAMLGEANN  305

Query  285  LFARYFGPGSTFDDLGTLGKELYATERMAEDRRSILIETESNFIHVLRRPQMIV  338
            +F   +GP        TLG+ELY  E   +    I  E  S  +    RPQ++V
Sbjct  306  IFEVAYGPCPKMGYANTLGQELYVFEYEKDRDEGIDFEAHSYMLPYCTRPQLLV  359
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Figure 5. Characterization of the PAK-P1 bacteriophage. *A*, Electron microscopic analysis of the PAK-P1 bacteriophage (scale bar, 100 nm). *B*, Sodium dodecyl sulfate gel of PAK-P1 bacteriophage proteins stained with coomassie blue (the arrow indicates the major capsid protein). *C*, Complete amino acid sequence of the major capsid protein. *D*, Basic Local Alignment Search Tool for Proteins (BLASTP) search result of the major capsid protein of the PAK-P1 bacteriophage (query) matching the major capsid protein of the Felix 01 bacteriophage (subject).

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of light emitted from mice in the bacteriophage-pretreated group was ~5 times lower than that emitted from mice in the buffer-pretreated group (Figure 4). Monitoring at the next time points confirmed that the bacterial load decreased in bacteriophage-pretreated animals and increased in buffer-pretreated animals (Figure 4). Finally, 100% of bacteriophage-pretreated animals survived until the end of the experiment (16 d), whereas 100% of untreated animals died within 2 d.

Genome sequence and characterization of the PAK-P1 bacteriophage. Electron microscopic observations revealed that the PAK-P1 bacteriophage is a member of the Myoviridae family, the same family as bacteriophage T4 of *Escherichia coli* (Figure 5) [13]. According to a recent proposal for a rational scheme for the nomenclature of viruses [24], this bacteriophage should be named "vB_PaeM_PAK_P1," which we have abbreviated as "PAK-P1" in this paper. Full genome sequencing of the PAK-P1 bacteriophage was performed. Compared with the genome sizes of the 38 fully sequenced *P. aeruginosa* bacteriophages, the size of the PAK-P1 bacteriophage genome (93,398 nucleotides, between the size of the LMA2 bacteriophage genome [66,530 nucleotides] and that of the EL bacteriophage genome [211,215 nucleotides]) suggested that it was a potentially new *P. aeruginosa* bacteriophage. The nucleotide sequence in the 6 frames was translated, and every open reading frame (ORF) $\geqslant$60 amino acids was used as a query for Basic Local Alignment Search Tool for Proteins (BLASTP) against the Classification of Mobile Genetic Elements (ACLAME) database [25, 26]. Results were scanned to match the following keywords: integrase, recombinase, repressor, and excisionase (considered as markers of temperate bacteriophages; see the Appendix, which appears only in the online version of the *Journal*). No statistically significant BLASTP similarity was identified. Consequently, the PAK-P1 bacteriophage should be considered as a virulent bacteriophage. We also analyzed the major capsid protein by means of mass spectrometry (Figure 5). An ORF of 344 amino acids (39.4 kDa) was then identified as the major capsid protein (Figure 5). A BLASTP search against the nr database (National Center for Biotechnology Information) revealed similarities with several putative bacteriophage-related proteins (the best result showed 33% identity with an *E* value of 4×10^{-41}), among which only 1 was annotated as a capsid protein of the Felix 01 bacteriophage (with 28% identity and an *E* value of 6×10^{-30}) (Figure 5). This result demonstrates that the PAK-P1 bacteriophage is composed of a novel major capsid protein. Taken together, these data confirm that the PAK-P1 bacteriophage is a new virulent bacteriophage of *P. aeruginosa*.

DISCUSSION

Our experiments demonstrate that noninvasive bioluminescence technology is remarkably useful for assessing the efficacy of bacteriophage treatment and especially for studying infection kinetics at early time points without sacrificing animals. For example, in the time course of the experiment to determine the optimal time for administering bacteriophage after the onset of infection, bioluminescence images helped us to understand why early bacteriophage inoculation (2 h after infection) was so important in resolving PAK infection. It is during this early infection stage (as confirmed by the lactate dehydrogenase levels) that the multiplication of bacteria is fastest. Under such conditions, susceptibility of bacteria to bacteriophage infection is also at its highest. Thus, infection is rapidly reduced with a reduction of the inflammatory response in the host, as shown by the levels of IL-6 and TNF-α. This is an advantage because excess inflammatory response can be harmful [21–23]. The rapid efficacy of bacteriophages in killing bacteria inside lungs suggests that there is no specific cellular factor (eg, proteases) that is active enough to prevent bacteriophages from infecting bacteria. Hence, it is anticipated that a bacteriophage determined to be efficacious in vitro might be efficacious in vivo. This hypothesis still needs to be proved, and if it were confirmed for the lungs it might not necessarily apply to other organs. Our data also agree with a mathematical model for bacteriophage therapy that was recently proposed by Cairns et al [27].

The PAK-P1 bacteriophage described in this paper was more efficacious against clinical strains isolated from patients with primary colonization than against strains from patients with chronic infection, which accords with the fact that the bacteriophage was isolated from planktonic cultures. In the case of chronic infection, the bacteriophage described here is probably not the most appropriate. For such situations more adequate bacteriophages should be specifically isolated, or existing bacteriophages could be "selected" by cultivating them on bacteria growing in biofilms to make them more efficacious [28, 29]. Hanlon et al [30] demonstrated that bacteriophages were active on *P. aeruginosa* biofilms made of alginates by taking advantage a depolymerase enzyme released by lysed bacteria.

Our choice to use a natural route for both infection and treatment allowed us to demonstrate that the respiratory tract from the upper parts to the lower parts could be treated with bacteriophages. More interestingly, we demonstrated that bacteriophages can actively prevent an infection from occurring and provide 100% protection when given 24 h before a deadly bacterial challenge. Our results showed that bacteriophages are not rapidly eliminated in the lungs. Such an effect was not anticipated, because it is generally reported that bacteriophages are quickly eliminated from the body [31]. This observation suggests that a preventive treatment might still be efficacious if bacteriophages are given 48 or 72 h before the start of infection, or if the amount of bacteriophages is decreased when given 24 h before infection. Such possibilities will expand the applications of bacteriophages to prevent infections from oc-

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curring. On the basis of the low level of cytokines induced by the bacteriophage solution, it is tempting to consider that the preventive effect is only due to bacteriophages rather than due to an inflammatory reaction. However, we cannot exclude the possibility that bacteriophages could induce phagocytic activity of cells such as macrophages toward bacteria (which may not necessarily require cytokine production). Infection of bacteria by in-site bacteriophages decreases rapidly the amount of pathogenic bacteria, reducing chances for the bacteria to start an active infection process and provoke lung damage. Recently, Golshahi et al [32] provided evidence that bacteriophages given by means of nebulization should be efficiently distributed in the lungs, which accords with the preventive effect we described. Taken together, these results clearly open the possibility of using bacteriophages in the prevention of bacterial lung infections. For example, one possibility is pretreatment of populations at risk for such infections (immunocompromised patients or patients with cystic fibrosis) to decrease the probability of infection in places where patients are more likely to be infected by bacteria (eg, care centers or hospitals). This is particularly relevant in situations in which an epidemic strain has been identified and for which preventive treatment with specific bacteriophages could be used to limit its spread. Such epidemic strains have been reported previously for *P. aeruginosa* in centers for treatment of cystic fibrosis [33]. Another situation in which a preventive treatment could be proposed is an influenza pandemic. According to recent studies, a predominant cause of death during the 1918 influenza pandemic was pneumonia rather than influenza itself [34]. A preventive bacteriophage treatment against pneumonia using a bacteriophage cocktail that targets the most prevalent lung pathogens could be envisaged in the case of a new influenza pandemic and could probably substantially decrease the number of deaths.

For obvious reasons, the genome sequence of a natural bacteriophage that could be considered for therapeutic use should be determined. However, complete annotation of a bacteriophage genome requires in-depth bioinformatics analysis, because gene-coding sequences found in bacteriophages are highly variable; moreover, few bacteriophage genomes have been analyzed in depth. Instead, here we propose a brief analysis aimed at confirming the virulent nature of the bacteriophage. Taking advantage of the ACLAME database, we determined that among all potential ORFs in the 6 reading frames, no significant match to proteins annotated as integrase, repressor, transposase, and excisionase was found. Because the purpose of the ACLAME database is to collect and annotate all proteins from mobile genetic elements, we assessed from our analysis that the PAK-P1 bacteriophage is not a temperate bacteriophage. Finally, the identification of the major capsid protein confirmed that the PAK-P1 bacteriophage is a new virulent bacteriophage of *P. aeruginosa*.

In conclusion, our work supports the potential use of bacteriophages to fight pathogens involved in lung infections and to develop an application to prevent such infections from occurring. Moreover, with the use of bioluminescent bacteria it is now possible to compare several bacteriophages (using a small number of animals) in order to establish a classification of candidates for therapeutics based on their real in vivo efficacy instead of their in vitro performance.

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References

1. Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infect Dis **2002**; 2:25–32.
2. Walsh C. Where will new antibiotics come from? Nat Rev Microbiol **2003**; 1:65–70.
3. Merril CR, Scholl D, Adhya SL. The prospect for bacteriophage therapy in Western medicine. Nat Rev Drug Discov **2003**; 2:489–497.
4. Brussow H. Phage therapy: the *Escherichia coli* experience. Microbiology **2005**; 151:2133–2140.
5. Barrow PA, Soothill JS. Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. Trends Microbiol **1997**; 5:268–271.
6. Smith HW, Huggins MB. Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. J Gen Microbiol **1983**; 129:2659–2675.
7. Sulakvelidze A, Kutter E. Bacteriophage therapy in humans. In: Kutter E, Sulakvelidze A, eds. Bacteriophages: biology and applications. Boca Raton, FL: CRC Press, **2005**:381–436.
8. Gorski A, Miedzybrodzki R, Borysowski J, et al. Bacteriophage therapy for the treatment of infections. Curr Opin Investig Drugs **2009**; 10: 766–774.
9. Hatfull GF. Bacteriophage genomics. Curr Opin Microbiol **2008**; 11: 447–453.
10. Campbell A. The future of bacteriophage biology. Nat Rev Genet **2003**; 4:471–477.
11. Summers WC. Bacteriophage therapy. Annu Rev Microbiol **2001**; 55: 437–451.
12. Pier G, Ramphal R. *Pseudomonas aeruginosa*. In: Mandell GL, Bennett JE, Dolin R, eds. Principles and practice of infectious diseases. 6th ed. New York, NY: Elsevier, **2004**:2587.
13. Ackermann HW. 5500 Phages examined in the electron microscope. Arch Virol **2007**; 152:227–243.
14. Wang J, Hu B, Xu M, et al. Use of bacteriophage in the treatment of experimental animal bacteremia from imipenem-resistant *Pseudomonas aeruginosa*. Int J Mol Med **2006**; 17:309–317.
15. Watanabe R, Matsumoto T, Sano G, et al. Efficacy of bacteriophage therapy against gut-derived sepsis caused by *Pseudomonas aeruginosa* in mice. Antimicrob Agents Chemother **2007**; 51:446–452.
16. Heo YJ, Lee YR, Jung HH, Lee J, Ko G, Cho YH. Antibacterial efficacy of phages against *Pseudomonas aeruginosa* infections in mice and *Drosophila melanogaster*. Antimicrob Agents Chemother **2009**; 53:2469–2474.

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17. Hutchens M, Luker GD. Applications of bioluminescence imaging to the study of infectious diseases. Cell Microbiol **2007**; 9:2315–2322.
18. Moir DT, Ming D, Opperman T, Schweizer HP, Bowlin TL. A high-throughput, homogeneous, bioluminescent assay for *Pseudomonas aeruginosa* gyrase inhibitors and other DNA-damaging agents. J Biomol Screen **2007**; 12:855–864.
19. Boulanger P. Purification of bacteriophages and SDS-PAGE analysis of phage structural proteins from ghost particles. Methods Mol Biol **2009**; 502:227–238.
20. Ackermann HW. Basic phage electron microscopy. Methods Mol Biol **2009**; 501:113–126.
21. Mukhopadhyay S, Hoidal JR, Mukherjee TK. Role of TNFα in pulmonary pathophysiology. Respir Res **2006**; 7:125.
22. Nonas SA, Finigan JH, Gao L, Garcia JG. Functional genomic insights into acute lung injury: role of ventilators and mechanical stress. Proc Am Thorac Soc **2005**; 2:188–194.
23. Adib-Conquy M, Cavaillon JM. Stress molecules in sepsis and systemic inflammatory response syndrome. FEBS Lett **2007**; 581:3723–3733.
24. Kropinski AM, Prangishvili D, Lavigne R. Position paper: the creation of a rational scheme for the nomenclature of viruses of *Bacteria* and *Archaea.* Environ Microbiol **2009**; 11:2775–2777.
25. Leplae R, Hebrant A, Wodak SJ, Toussaint A. ACLAME: a classification of mobile genetic elements. Nucleic Acids Res **2004**; 32:D45–D49.
26. Ladunga I. Finding homologs in amino acid sequences using network BLAST searches. Curr Protoc Bioinformatics **2003**; chap 3:unit 3.4.
27. Cairns BJ, Timms AR, Jansen VA, Connerton IF, Payne RJ. Quantitative models of in vitro bacteriophage-host dynamics and their application to phage therapy. PLoS Pathog **2009**; 5:e1000253.
28. Knezevic P, Petrovic O. A colorimetric microtiter plate method for assessment of phage effect on *Pseudomonas aeruginosa* biofilm. J Microbiol Methods **2008**; 74:114–118.
29. Sillankorva S, Neubauer P, Azeredo J. *Pseudomonas fluorescens* biofilms subjected to phage phiIBB-PF7A. BMC Biotechnol **2008**; 8:79.
30. Hanlon GW, Denyer SP, Olliff CJ, Ibrahim LJ. Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa* biofilms. Appl Environ Microbiol **2001**; 67:2746–2753.
31. Skurnik M, Strauch E. Phage therapy: facts and fiction. Int J Med Microbiol **2006**; 296:5–14.
32. Golshahi L, Seed KD, Dennis JJ, Finlay WH. Toward modern inhalational bacteriophage therapy: nebulization of bacteriophages of *Burkholderia cepacia* complex. J Aerosol Med Pulm Drug Deliv **2008**; 21:351–360.
33. Armstrong D, Bell S, Robinson M, et al. Evidence for spread of a clonal strain of *Pseudomonas aeruginosa* among cystic fibrosis clinics. J Clin Microbiol **2003**; 41:2266–2267.
34. Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis **2008**; 198:962–970.
35. Balloy V, Verma A, Kuravi S, Si-Tahar M, Chignard M, Ramphal R. The role of flagellin versus motility in acute lung disease caused by *Pseudomonas aeruginosa.* J Infect Dis **2007**; 196:289–296.

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